Reference: *Biol. Bull.*, **150**: 500–521. (June, 1976)

ACTIVATORY MECHANISMS OF THE PROTHORACIC GLANDS OF *MONEMA FLAVESCENS* (LEPIDOPTERA) WITH SPECIAL REFERENCE TO THE SECRETION OF ECDYSONE

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It had been assumed that the prothoracic glands synthesize and secrete ecdysone in the manner of an endocrine gland, despite the lack of direct experimental evidence, until a first preliminary report that the prothoracic glands can secrete ecdysone *in vitro* appeared (Takeda, 1972a). This classical theory came basically from the experimental morphological findings that the insects into which active prothoracic glands were implanted were induced to molt (Fukuda, 1940, 1944). The amount of secreted hormone has always been too small to provide proof that this gland is the site of ecdysone synthesis.

It is well known that α-ecdysone was isolated first from *Bombyx mori* (Butenandt and Karlson, 1954), synthesized from cholesterol (Karlson and Hoffmeister, 1963; Galbraith, Horn, Middleton, Thomson and Thomson, 1970; Moriyama, Nakanishi, King, Okauchi Siddall and Hafferl, 1970; Gersch and Stürzebecher, 1971; Willig, Rees and Goodwin, 1971; Nakanishi, Moriyama, Okauchi, Fujioka and Koreeda, 1972; Studinger and Willig, 1975) and is rapidly metabolized to β-ecdysone (King and Siddall, 1969; Moriyama *et al.*, 1970; Nakanishi *et al.*, 1972). The possibility was reported that the prothoracic glands secrete into the hemolymph an enzyme necessary for ecdysone biosynthesis from cholesterol (Gilbert, 1964; Bern, 1967). Locke (1969) and Weir (1970) reported that ecdysone may be secreted from the oenocyte. Further, Romer (1973) extracted a considerable amount of ecdysones from isolated oenocytes of *Tenebrio molitor*. Moriyama *et al.* (1970) found α-ecdysone synthesis in the abdomen of *Bombyx mori* which did not contain the prothoracic glands. They postulated that the prothoracic gland hormone was not identical to ecdysone but acts to transform the inactive-bound ecdysone to the active form. Therefore, a possibility arises that the prothoracic glands are the source of a second trophic hormone rather than ecdysone. Since no prothoracic glands are present in the isolated abdomen, a doubt is cast on the long standing principle in insect endocrinology that the prothoracic glands are the source of ecdysone synthesis. Nakanishi *et al.* (1972) postulated strongly that the prothoracic glands act only as a source of an enzyme necessary for the biosynthesis of ecdysone from cholesterol.

Recent *in vitro* work, however, supports the original notion that the prothoracic glands do synthesize ecdysones or substances with ecdysone-like activity. Willig *et al.* (1971) reported that isolated ring gland-brain complexes of the blowfly, *Calliphora erythrocephara,* incubated with cholesterol, synthesized ecdysone, most often in the form of glycoside and esters. It was also found that spermatogenesis in the isolated testes of diapausing silkworm pupa, *Hyalophora cecropia,* incubated in a cell-free hemolymph was stimulated *in vitro* by the addition of activated pro-

thoracic glands or by inactive glands together with active brain or ecdysone. However, inactive glands or active brain alone produced no response (Kambysellis and Williams, 1971, 1972). It was ascertained that the prothoracic glands taken from six-day old larvae in the final instar of the cabbage armyworm, *Manestra brassicae,* released a hormonal agent which induced the molting of the integument of the rice stem borer, *Chilo suppresalis* (Agui, Kimura and Fukaya, 1972). More recently, other research groups have provided some chemical evidence that the cultured prothoracic glands of the silkworm, *Bombyx mori* (Chino, Sakurai, Ohtaki, Ikekawa, Miyazaki, Ishibashi and Abuki, 1974), the tobacco hornworm, *Manduca sexta* (King, Bollenbacher, Borst, Vedeckis, O'Connor, Ittycheriah and Gilbert, 1974), the cockroach, *Leucophaea maderae* (Borst and Engelmann, 1974; King and Marks, 1974) and the mealworm, *Tenebrio molitor* (Romer, Emmerich and Nowock, 1974) produce α-ecdysone.

Although the physiological properties of ecdysone have been extensively studied (Novák, 1966; Šorm and Sláma, 1974), the synthetic site of ecdysone has not been clear. It waxes and wanes under the direction of the central nervous system which intermittently releases a tropic hormone referred to as the prothoracotropic hormone (brain hormone), and finally regresses following the terminal molt on the diapause break (Fukuda, 1944; Williams, 1947, 1952). In *Monema flavescens,* the neurosecretory substances, the prothoracotropic hormones, are released from the neurosecretory B-cells in the *pars intercerebralis* (Takeda, 1972b). These substances migrate directly across the perineurium to the hemolymph (Takeda, 1976). Then, they are transported to the prothoracic glands by the granular hemocytes and activate them (Takeda, 1971a).

On the other hand, many papers on the histology of the prothoracic glands have appeared (Herman, 1967). However, knowledge of the prothoracic glands in relation to the secretion seems to be small (Beaulaton, 1964, 1968; Herman and Gilbert, 1966; Yashika and Yoshizaki, 1967). For these reasons, the problems of secretion in the prothoracic glands with special reference to ecdysone synthesis are investigated in this study.

Materials and Methods

Materials used in this study were the slug moth prepupa, *Monema flavescens* Walker (Lepidoptera: Heterogeneidae). Specimens were collected at the diapause period in the field and placed under natural conditions. When each experiment was carried out, parts of the materials were incubated in order to estimate the degree of diapause intensity (Takeda, 1971b).

For light-microscopic studies, the head regions were fixed with Bouin, Susa, Susa-picric and Kahle's methods modified by Huttner for 24 hours, respectively, at intervals from September to the following March. They were dehydrated in butyl alcohol, embedded in paraffin wax and sectioned at 3 μ. Staining methods applied in these experiments were Hematoxylin-eosin, Mallory's triple stain, Gabe's chromalum hematoxylin phyloxin, Clark's aldehyde fuchsin and $HgCl_2$ bromphenol blue.

For electron-microscopic studies, the prothoracic glands were quickly extirpated under a dissecting microscope, then immediately pre-fixed in cold 2.5% glutaraldehyde (pH 7.4: phosphate buffer) for ninety minutes and were post-fixed in cold 2% osmium tetraoxide (pH 7.4: Veronal buffer) for three hours. They were passed

through cold 50%, 70%, and 90% ethyl alcohol for 45 minutes at room temperature. After dehydration, they were placed in absolute acetone for one hour and were gradually infiltrated with Epon 812 (Luft, 1961). The Epon blocks were trimmed and sectioned using an ultramicrotome with glass knives. The staining was performed with lead citrate (Reynolds, 1963). Micrographs were taken with JEM-T6S electron microscopy.

For autoradiography, tritiated uridine was employed at a concentration 1 mCi/mM with a specific activity 1.9 mCi/mM. All insects were killed several hours after the injection. They were embedded in paraffin wax and cut at 3 μ. Serial sections were coated with Sakura nuclear-track emulsion, NR-M2. The coated slides were exposed for four weeks at 0° C. All slides were developed at 20° C for one minute in Konidol X (Sakura). Autoradiographs were stained with Mayer's hemalum-fast green.

Histochemical tests for cholesterol were carried out in the prothoracic glands fixed with formalin. The tissues were then treated with 0.5% digitonin in ethyl alcohol (50%) for ten days, and then with ethyl alcohol and ether for three hours. After that, Lieberman-Schultz's methods for cholesterol were applied to the frozen sections obtained by cryostat.

For another autoradiography, ^{14}C-cholesterol (^{14}C-cholesterol-4-C: specific activity, 33.5 mCi/mM) was employed at a concentration of 1 mCi/ml. A dosage of 1 μCi/1 g of body weight was injected into the abdomen with a microsyringe. After several hours (12, 24 and 48 hours), insects were fixed with Bouin containing saturated digitonin. Digitonin is known to make an unsoluble complex in some organic solvents reacting with isolated cholesterol. Therefore, this treatment is known to make it easy to capture cholesterol. Sections were placed on glass slides and coated with autoradiography emulsion (Sakura: NR-M2). After three weeks exposure, the slides were developed with Konidol X (Sakura) at 20° C for five minutes and stained with Mayer's hematoxylin and eosin. The uptake of cholesterol into the prothoracic glands and the migration of the grains to the hemolymph were examined by counting the grains on two slides per insect and four sections per slide. All grain counts were made under oil immersion at 1250×. Background was less than two grains per 100 μ^2 in all cases and was not subtracted from grain counts. Grain counts were performed in the cytoplasm of the prothoracic glands and the area of hemolymph per 100 μ^2, respectively.

Ecdysone biosynthesis in the prothoracic glands *in vivo* was examined. ^{14}C-cholesterol dissolved in linolenic acid (4.2 μCi ^{14}C-cholesterol/2.5 μl linolenic acid/prepupa) was injected into the diapausing and post-diapausing prepupae, respectively. In other experiments, prepupae were ligated with thread and divided into two parts, the prior part with and the posterior part without prothoracic glands. In each part, ^{14}C-cholesterol was injected at half the previous dosage. In these experiments, after several hours, the parts were homogenized and extracted four times with hot ethyl alcohol (60–70° C) over 24 hours. The extracts were evaporated and the residues dissolved in 20% ethyl alcohol for column chromatography to analyze ecdysone biosynthesis.

Ecdysone biosynthesis in the prothoracic glands also was examined *in vitro*. Thirty pairs of the prothoracic glands of diapausing and post-diapausing prepupae were cultured respectively in the medium CSM-2F (Mitsuhashi, 1968). A small amount of ^{14}C-cholesterol (0.02 mM) was added at the onset of cultivation. After

several days, the medium was extracted with hot alcohol, and the residue was dissolved in 20% ethyl alcohol for column chromatography to analyze ecdysone biosynthesis. In some parts, ecdysone biosynthesis was also studied in the prothoracic glands themselves after removing the culture medium by the same methods.

To detect ecdysones, liquid chromatographs were used according to Hori (1969), employing Amberlite XAD-2 (40–50 mesh) and sigmoid gradient elution with 20–90% ethyl alcohol in water at 20° C. As the amount of ecdysones involved in *Monema flavescens* was minute, the fractions of ecdysones were determined at first by the application of pure ecdysones, α-ecdysone and β-ecdysone. The fractions of α- and β-ecdysones were determined by a photometer at 254 mμ after chromatographying with the application of pure α- and β-ecdysones, respectively, before experiments. The fractions of α- and β-ecdysones corresponded to fraction numbers 41 and 51, respectively.

After application of the sample, each fraction was prepared for a scintillation counter. Scintillation fluid consisted of naphthalene 180 g, POPOP 0.5 g, PPO 9 g, ethylene glycol monoethyl ether 500 cc and dioxane 3000 cc. Radioactivity of each fraction was counted with a liquid scintillation counter (Packard).

Although it was ascertained photometrically that α-and β-ecdysones corresponded to fractions 41 and 51, respectively, bioassay of these fractions was performed further. The samples were obtained by chromatography from the medium-cultured prothoracic glands of post-diapausing prepupae for five days with addition of nonlabelled cholesterol at the onset of cultivation. The solution of fractions 41 and 51 was concentrated by the evaporator and injected into the diapausing prepupae. After seven days, the state of the insects was observed and compared to controls.

Results

Histological observations of the prothoracic glands with the progress of diapause-break

To clarify the secretion of ecdysone in the stage of diapause-break, the histological changes found in the prothoracic glands with the progress of diapause-break were examined.

Throughout the diapause period, the appearance of the prothoracic gland was almost unchanged. The nucleus was usually regularly spherical or in the shape of a slightly flattened ellipsoid. It appeared to be enclosed in a closely applied membrane, whereas intra-or inter-cellular material was not apparent. The gland cells were spheroid, possessed relatively regular nuclei and inconspicuously striated borders (Fig. 1). The fine structure of the gland cells in the diapause stage is shown in Figure 6. The development of granular-ER was not found. However, many free ribosomes were seen. Lipid droplets and glycogen particles were also seen. In many parts of the gland cells, the presence of undeveloped agranular-ER was clearly observed. This seemed to indicate that the glands have lower activity in this stage and that the synthesis of the secretory substances was suppressed. With the progress of diapause break, striking changes appeared. Initial secretory activity was characterized by the increased nuclear and nucleolar volume that preceded the appearance of the nuclear irregularity. The most striking cytological feature of

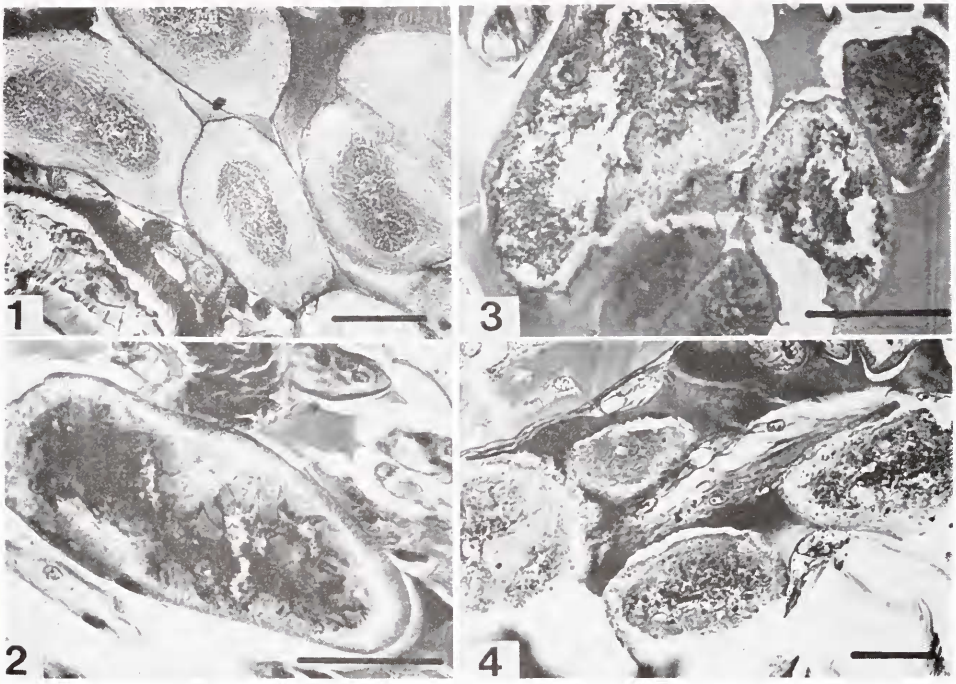


FIGURE 1. Prothoracic glands in the diapausing stage; scale 25 μ.
FIGURE 2. Prothoracic gland at the beginning of nuclear folding; scale 25 μ.
FIGURE 3. Nuclear folding found in the prothoracic glands; scale 25 μ.
FIGURE 4. Prothoracic glands which began to synthesize the secretory substances in the cytoplasm; scale 25 μ.

these glands was the nuclear folding. Figure 2 and Figure 3 show the process of nuclear folding with the progress of diapause-break. Under electron microscopy, the nucleus was enveloped by a double-layered nuclear membrane and contained a few nucleolei, while in the diapause stage, the nucleus was rounded and the folding

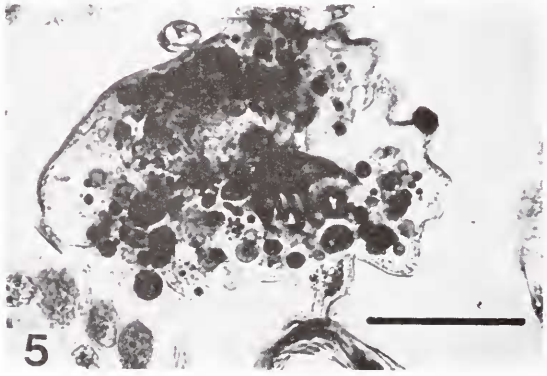


FIGURE 5. Prothoracic gland containing many secretory substances in the cytoplasm; scale 25 μ.

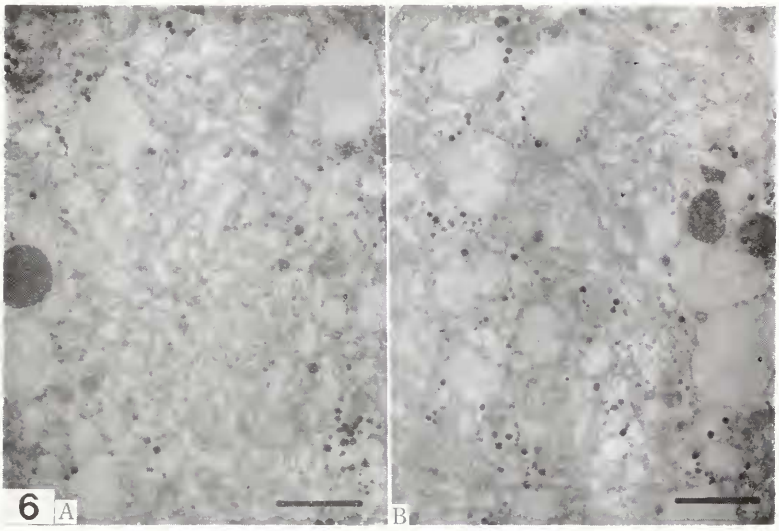


FIGURE 6. (A and B) General views of cytoplasm of the prothoracic gland in the diapausing stage. Undeveloped agranular-ER, lipid droplets, glycogen particles and free ribosomes etc. are seen; scale 1 μ.

did not appear (Fig. 7A). With the progress of the cyclic activity in the gland, the nucleus contained numerous chromatin blocks and few nucleolei and became irregular (Fig. 7B). Then the nucleus began to branch out into the cytoplasm (Fig. 7C). The nuclear folding seems to have an important role in the interaction between the nucleus and the cytoplasm. In this stage, the information necessary for the synthesis of the secretory substances must be transferred from the nucleus to the cytoplasm. After passing this stage, secretory substances began to appear in the cytoplasm of the prothoracic glands (Fig. 4). The secretory substances gradually became large in size and finally the cytoplasm was filled with the secretory substances (Fig. 5). Although the releasing mechanisms were unclear, they were released from the cell to the hemolymph. Agranular-ER, which is believed to synthesize the steroid hormone, began to develop in the peripheral region with the progress of nuclear folding. They were arranged to encircle a relatively narrow intracellular space and formed randomly interconnecting tubes in the peripheral region (Fig. 8). With the progress of the development of agranular-ER, the contact figures between agranular-ER and mitochondria were observed in some places (Fig. 9). This seemed to indicate the transfer of some substances from the agranular-ER to the mitochondria. After that, the deformation of mitochondria was seen in some parts of the cytoplasm. The deformation coincided well with the schematic drawings provided by Wilde (1969). At first, mitochondria were swollen with less dense interiors and their cristae became unclear. Then these organelles were more swollen and some granules formed in them. Thereafter, the number of normal mitochondria decreased gradually and deformed mitochondria made up the greater part of the total mitochondria. This is shown in Figure 10. In the secreting stage, there appeared many secretory granules in the cytoplasm. They may have originated from the mitochondria whose surface membrane had disappeared (Fig. 11). They

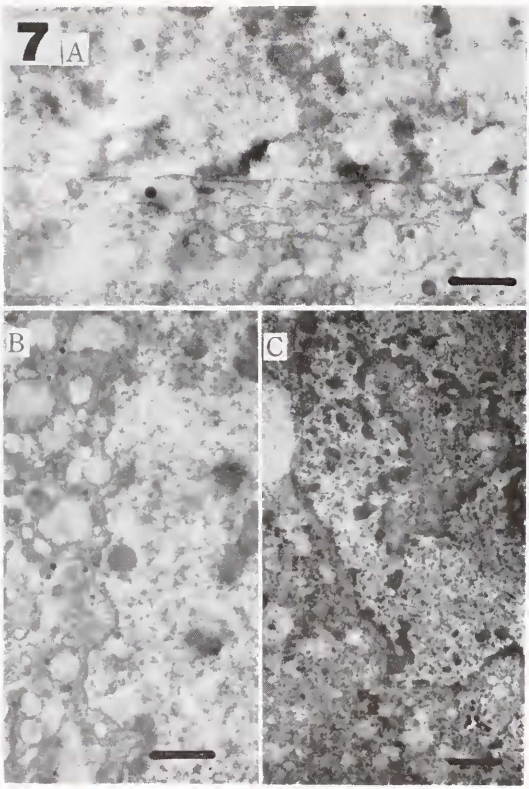


FIGURE 7. A, Appearance of nuclear folding found in the prothoracic glands. The nucleus is rounded and the folding does not appear in the diapausing stage; B, nuclear surface becomes irregular with the progress of activation; C, nucleus then begins to branch out into the cytoplasm; scale 1 μ.

appeared lamellar, granular or nonstructured. Under low magnification, one of them had the appearance of an array of slender filaments more or less parallel to each other. Higher magnification demonstrated that they were actually bundles of minute straight tubules. Nonstructured granules, however, were also present and they occupied the greater part of the secretory substances. From the present figures, the precise relationship between the secretory granules and ecdysone could not be deduced.

Studies were then made of the cell membrane regarding the release of these substances. The peripheral cell membrane of the prothoracic glands showed a form of microvilli (Fig. 12A) and through them the secretory substances seemed to be released. In the releasing stage, some substances always appeared in the peripheral region of the cytoplasm and further in the microvilli (Fig. 12B). The release of these substances was not observed in an intact form. Therefore, some substances found near the microvilli seemed to be the ones synthesized in the mitochondria. It is likely that the intact secretory substances collapsed and were released into the hemolymph through the microvilli in the releasing stage.

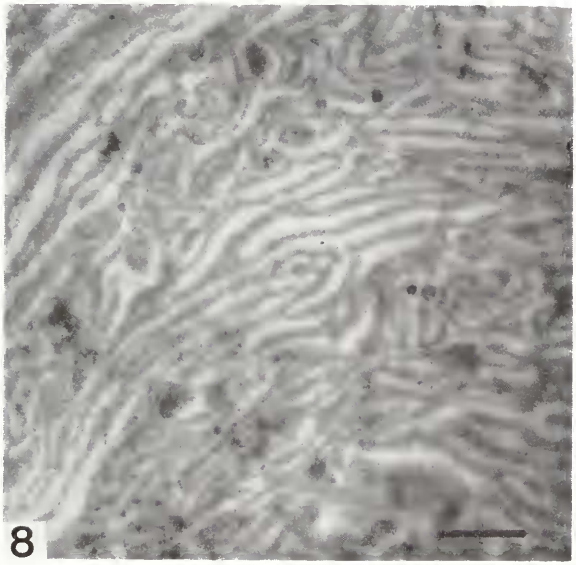


FIGURE 8. Central part of the well developed agranular-ER usually found in the peripheral region in the cytoplasm; scale 1 μ.

The incorporation of tritiated uridine to the prothoracic glands was then examined to clarify the relationship between glandular activity and uridine incorporation. The synthesis of RNA in the prothoracic glands of diapausing prepupae was

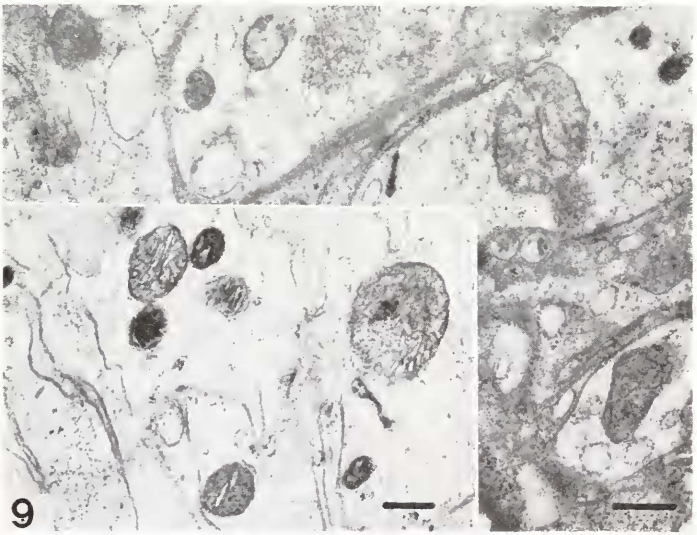


FIGURE 9. Contact figures found between agranular-ER and mitochondria. These are always found in the edge of agranular-ER, away from the central area. Tubules are distinct, different from those of the swollen agranular-ER found in the central part of the mass of agranular-ER; scale 1 μ.

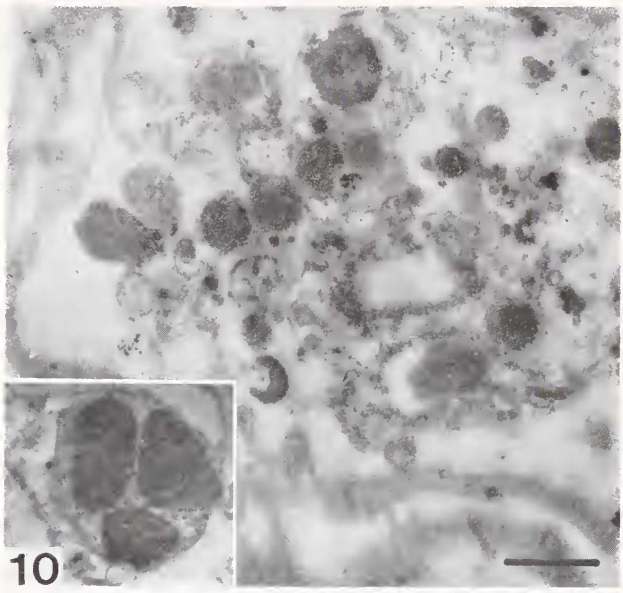


FIGURE 10. Secretory substance synthesis found in the deformed mitochondria; scale 1 μ. Most of the mitochondria found in this stage appeared thus, and resembled the mitochondrial deformation reported by Wilde (1969) for the Colorado beetle.

not found until some factors such as brain hormone activated the glands to secrete ecdysone. When the prothoracic glands were switched from quiescence to secretory activity, there was an increase in the rate of RNA synthesis. The initial low rate

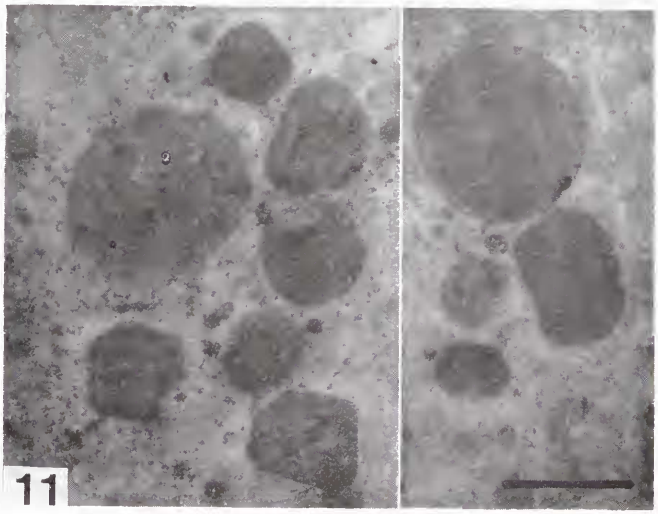


FIGURE 11. Secretory substances found in the cytoplasm. They appear in the cytoplasm after breaking the mitochondrial sheath; scale 1 μ.

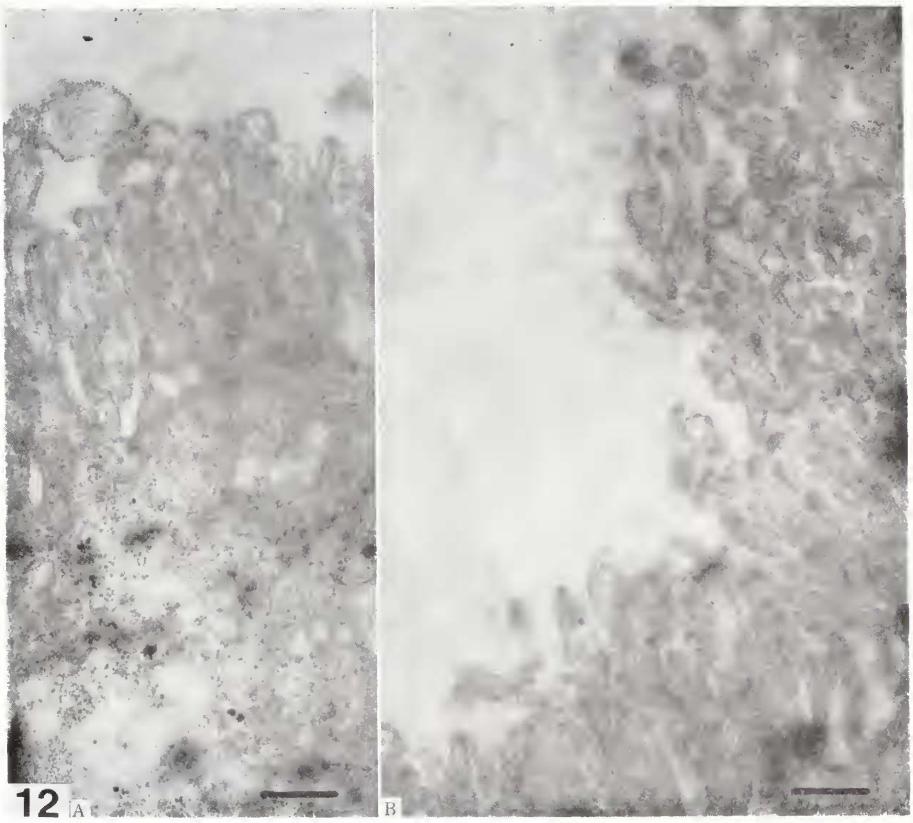


FIGURE 12. A, Microvilli found in the peripheral membrane of the prothoracic glands; B, release of the secretory substances from the microvilli by pinching off tips of microvilli. Dark material to the right of B is made up of condensed secretory substances; scale 0.5 μ.

of RNA synthesis occurred at the time when the prothoracic gland-cell nuclei were regular in shape. By the time the maximum rate of RNA synthesis was reached, nuclear folding and vacuolization were evident. The incorporation was found first in the nucleus (Fig. 13) and then in the cytoplasm (Fig. 14). The correlation between RNA synthesis and the cytological changes during secretion described above was most striking. The increase of uridine incorporation was closely correlated with the appearance of secretory activity in the gland cells. It seems apparent that a close interaction between the nucleus and the cytoplasm is necessary for the synthesis of the secretory substances.

Ecdysone biosynthesis in the prothoracic glands

Detection of cholesterol in the prothoracic glands. Cholesterol has been demonstrated to be the precursor of ecdysone in *Calliphora erythrocephara* (Karlson and Hoffmeister, 1963; Willig *et al.*, 1971), *Calliphora stygia* (Galbraith *et al.*, 1970), *Bombyx mori* (Moriyama *et al.*, 1970; Nakanishi *et al.*, 1972), *Mamestra brassicae*

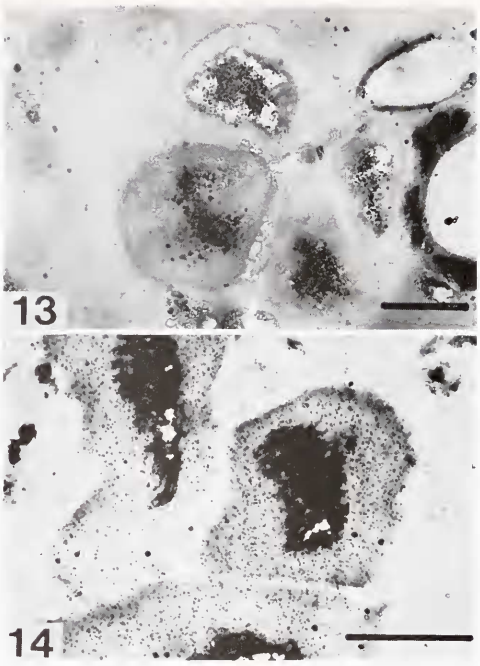


FIGURE 13. Incorporation of ^{3}H-uridine into the nucleus of the prothoracic glands; scale 25 μ.

FIGURE 14. Migration of labelled substances from the nucleus to the cytoplasm; scale 25 μ.

(Gersch and Stürzebecher, 1971), and *Musca domestica* (Studinger and Willig, 1975). Whether the prothoracic glands contained cholesterol was first examined. In *Monema flavescens,* the prothoracic glands showed a positive reaction in the part of their peripheral region (Figs. 16 and 17). As mentioned above, this area coincided with part of the well-developed agranular-ER, which had been shown to contain some enzymes necessary for steroid hormone synthesis from cholesterol (Shikita and Tamaoki, 1964; Clayton, 1969). Positive results were also found in the prothoracic glands of both diapausing and post-diapausing prepupae. It was more prominent in the post-diapausing stage (Fig. 17) than that of the diapausing stage (Fig. 16). The reaction was not uniform in the cytoplasm but always localized in the peripheral region of the gland cells. No detectable cholesterol was found in the muscle tissues used as controls (Fig. 15). The existence of cholesterol is highly significant for ecdysone biosynthesis.

Incorporation of ^{14}C-cholesterol into the prothoracic glands. Since insects are incapable of synthesizing cholesterol, cholesterol found in the prothoracic glands originates in food. The incorporation of cholesterol from the hemolymph to the prothoracic glands was examined by the autoradiography of ^{14}C-cholesterol. In the prothoracic glands of diapausing prepupae, the incorporation was not found, while it was clearly detected in post-diapausing prepupae (Fig. 18). These results were compared further with that of the secretory substance synthesis with the progress of diapause break. With the progress of secretory substance synthesis,

the incorporated grains which spread in the cytoplasm gathered each other and formed large grains which corresponded to the secretory substance (Fig. 19) and were released to the hemolymph (Fig. 20). The grains which appeared in the cytoplasm migrated to the hemolymph. They were not released from the cytoplasm in intact clusters of grains, but in small grains (Fig. 20). This agreed with the results of electron microscopy. The time course obtained by counting the grains also showed the results of migration clearly. The amount of grains in the hemolymph increased gradually with the decrease of the grains in the cytoplasm. It is unclear whether the large grains made up of small grains corresponded to the cholesterol molecules or to ecdysone. These results show that cholesterol is incorporated into the prothoracic glands from the hemolymph, and then is secreted into the hemolymph as small grains in response to need for ecdysone.

Ecdysone biosynthesis in the prothoracic glands in vivo. Ecdysone biosynthesis was examined at first in the intact body of the diapausing and post-diapausing prepupae after being injected with ^{14}C-cholesterol. In the diapausing prepupae, ecdysone biosynthesis was not found (Fig. 21, open circle). On the contrary, in the post-diapausing prepupae, high radioactivity was found in the fractions 41 and 51 which were demonstrated to be β-ecdysone and α-ecdysone, respectively (Fig. 21, closed circle). In *Monema flavescens,* the existence of both α- and β-ecdysones were clearly demonstrated *in vivo.*

To examine the synthetic site of ecdysones, ligature treatments were then carried out in the post-diapausing prepupae (Fig. 22). Diapausing prepupae were also used as controls. In the anterior part with the prothoracic glands, α- and β-ecdysones were clearly biosynthesized (Fig. 22, open circle). The amount of β-

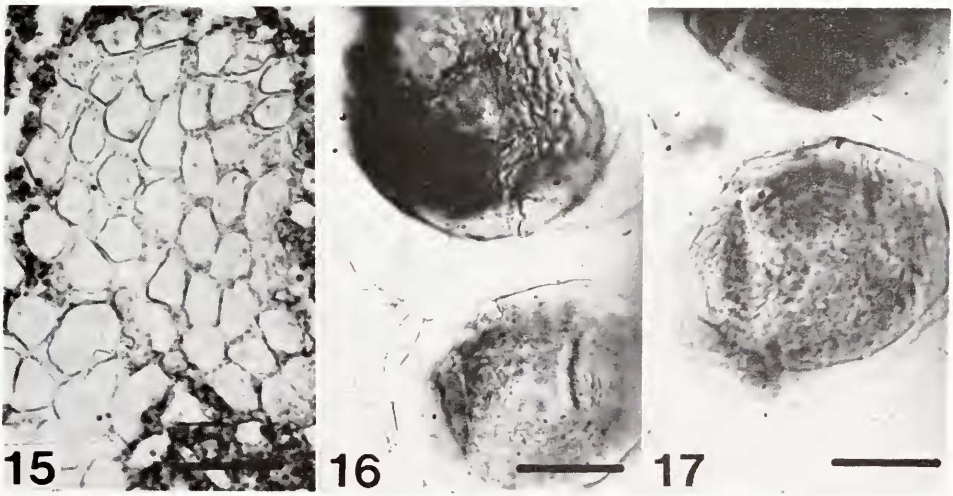
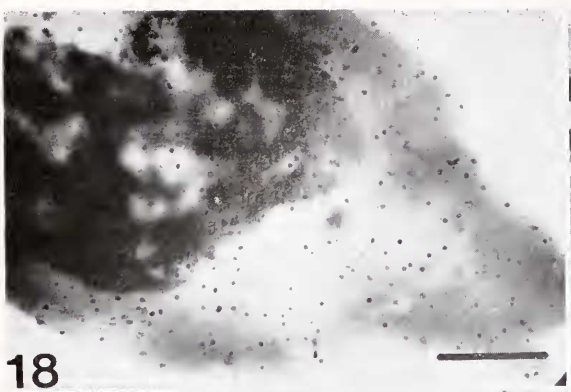
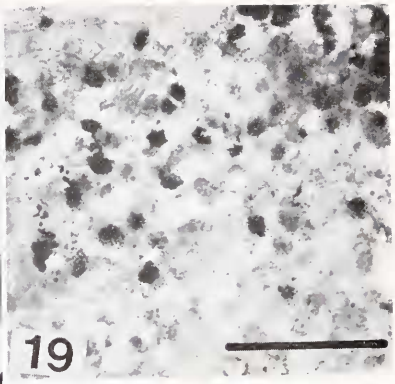


FIGURE 15. Histochemical test for cholesterol in muscle tissue (control). No positive reaction occurs in the whole area of the tissue; scale 25 μ.

FIGURE 16. Histochemical test for cholesterol in the prothoracic glands of diapausing stage. Positive reactions are found in the dark-stained peripheral region of the glands; scale 25 μ.

FIGURE 17. Histochemical test for cholesterol in the prothoracic glands of the post-diapausing stage. Positive reactions are again found in the peripheral region of the glands; scale 25 μ.





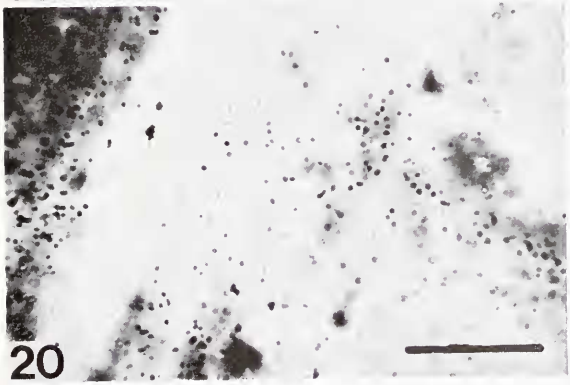


FIGURE 18. Incorporation of ^{14}C-cholesterol into the cytoplasm of the prothoracic glands. Grains are spread throughout the cytoplasm at the beginning of incorporation; scale 10 μ.

FIGURE 19. Clusters of labelled substances appear in the peripheral region of the cytoplasm, and represent the secretory granules; scale 25 μ.

FIGURE 20. Migration of labelled substances from the prothoracic glands (left) to the hemolymph; scale 10 μ.

ecdysone was more than that of α-ecdysone. The radioactivity of ecdysones was not detected in either the anterior part with the prothoracic glands or the posterior part without the prothoracic glands in the diapausing prepupae. Similar results were found in the isolated abdomens of *Bombyx mori* (Moriyama *et al.*, 1970; Nakanishi *et al.*, 1972), *Mamestra brassicae* (Gersch and Stürzebecher, 1971), and *Musca domestica* (Studinger and Willig, 1975). These experiments showed that ecdysone biosynthesis occurred not only in the prothoracic glands but also in the peripheral tissues without prothoracic glands. However, most ecdysones were biosynthesized in the prothoracic glands.

Ecdysone biosynthesis in the prothoracic glands in vitro. To examine the results in the former section, more direct experiments were performed *in vitro*. At first, the prothoracic glands of the post-diapausing stage were cultured in the synthetic medium CSM-2F for five days with the addition of labelled cholesterol at the onset of cultivation. The same treatment was also performed in the prothoracic glands of the diapausing stage as a control. After cultivation, ecdysone biosynthesis

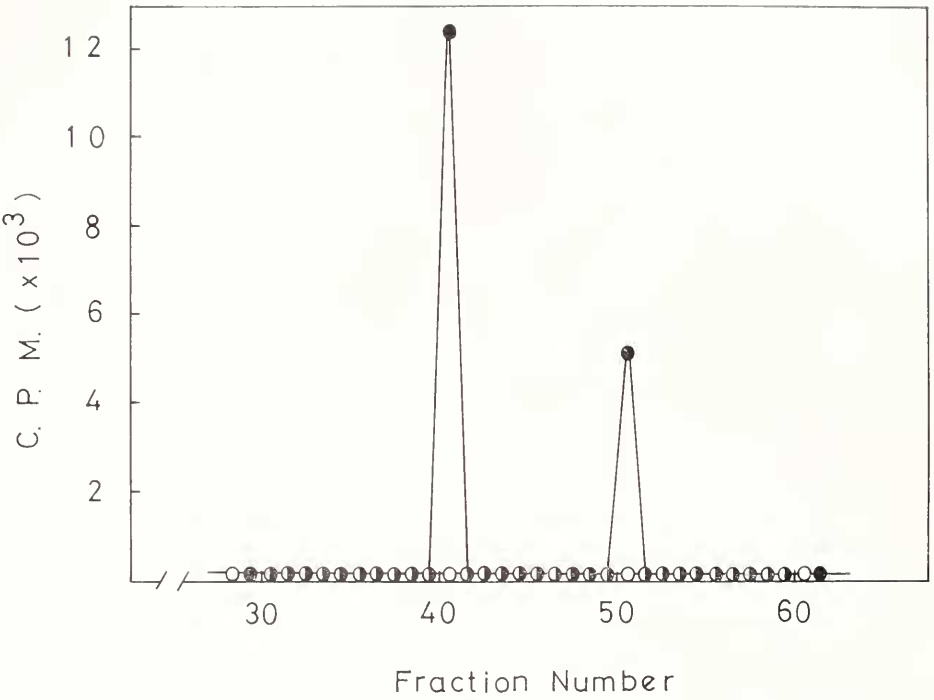


FIGURE 21. Ecdysone biosynthesis in the intact diapausing and post diapausing prepupae. Open circles indicate diapausing prepupae; closed circles, post diapausing prepupae. Fractions 41 and 51 fs correspond to β- and α-ecdysones, respectively. In the post-diapausing prepupae α- and β-ecdysones were clearly biosynthesized *in vivo*.

was examined. In the culture medium of the prothoracic glands of the diapausing stage, ecdysones were not biosynthesized completely. This was not changed by extending the term of cultivation to two weeks. On the other hand, α- and β-ecdysones were clearly biosynthesized in the culture medium of the prothoracic glands of the post-diapausing stage after the same number of days of cultivation (Fig. 23). The results provide the most direct evidence that the prothoracic glands produce and secrete ecdysones. The amount of α-ecdysone was more than that of β-ecdysone, differing from the results *in vivo*. However, in other insects, for example *Bombyx mori* (Chino *et al.*, 1974), *Manduca sexta* (King *et al.*, 1974), and *Leucophaea maderae* (Borst and Engelmann, 1974; King and Marks, 1974), β-ecdysone was not found in the culture medium. The reasons for this are not clear.

Ecdysone biosynthesis and storage was examined further in the prothoracic glands themselves. The prothoracic glands were cultured for five days with the addition of ^{14}C-cholesterol at the onset of cultivation. After that, ecdysone biosynthesis was examined. Before preparation, the prothoracic glands were washed several times in the culture medium CSM-2F. In both the prothoracic glands of diapausing and post-diapausing prepupae, ecdysones were not accumulated in the prothoracic glands themselves. Therefore, it seems likely that the incorporation

TABLE I

Bioassay of the active fractions on the diapausing prepupae of Monema flavescens *seven days after onset of treatment.*

Prothoracic glands	Active fractions	Post-diapausing prepupae (unaffected)	Prepupal-pupal intermediates	Pupae obtained
Post-diapausing stage	41 (β-ecdysone)	0	7	8
	51 (α-ecdysone)	0	13	2
Diapausing stage	41 (β-ecdysone)	6	0	0
	51 (α-ecdysone)	6	0	0
Controls	ethanol	10	0	0

of cholesterol is elaborated to α-ecdysone just before secretion. As α-ecdysone is known to convert into β-ecdysone, β-ecdysone found in the medium seems to originate from α-ecdysone. In the crab, *Hemigrapsus nudus,* however, "labelled β-

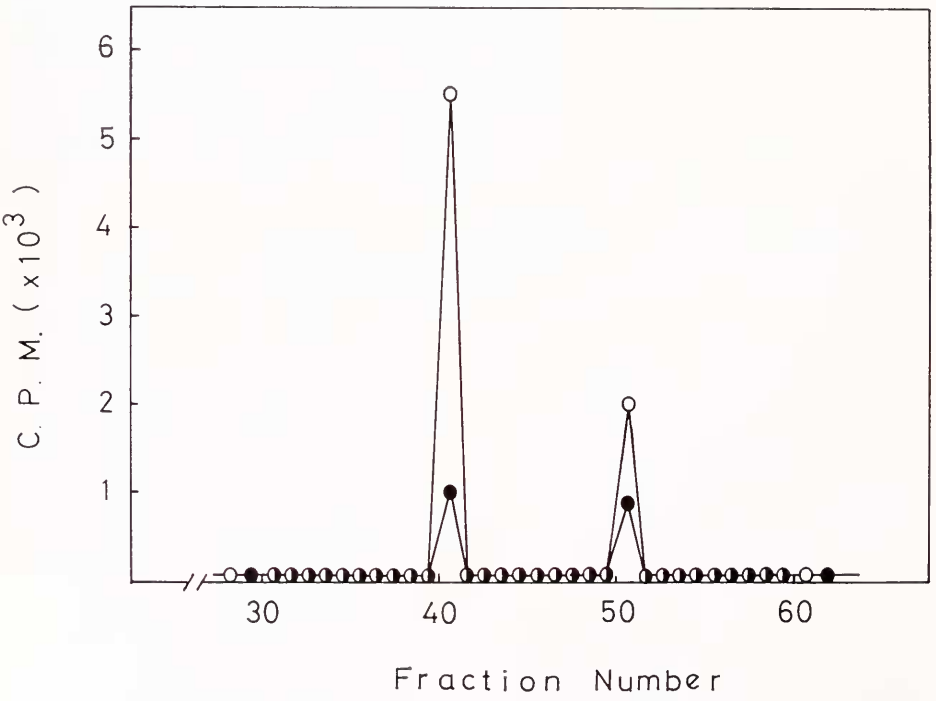


FIGURE 22. Ecdysone biosynthesis in the ligatured post diapausing prepupae. Open circles indicate prior part with prothoracic glands; closed circles, posterior part without prothoracic glands. Fractions 41 and 51 correspond to β- and α-ecdysones, respectively. Even in the posterior part without prothoracic glands, α- and β-ecdysones were biosynthesized *in vivo.*

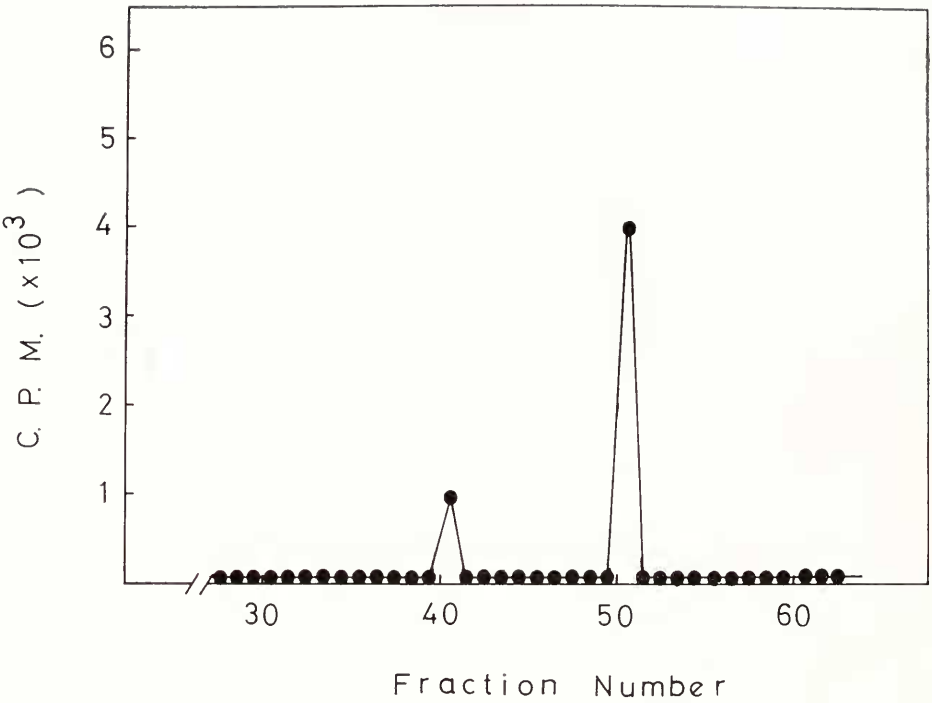


FIGURE 23. Ability of ecdysone biosynthesis in the prothoracic glands of post-diapausing prepupae *in vitro*. Fractions 41 and 51 correspond to β- and α-ecdysones, respectively. Closed circles indicate ecdysone biosynthesis *in vitro*. α- and β-ecdysones were clearly biosynthesized *in vitro*.

ecdysone" was found in the Y-organ itself (Spaziani and Kater, 1973). As described above, α-ecdysone is known to convert into β-ecdysone in *Leucophaea maderae* (King and Siddall, 1969) and in *Bombyx mori* (Moriyama *et al.*, 1970; Nakanishi *et al.*, 1972). In the present experiments, both α- and β-ecdysones were found. However, the amount of β-ecdysone was greater *in vivo* and less *in vitro* than that of α-ecdysone. This seems to result from their conversion.

Bioassay of active fractions. The bioassay of the active fractions obtained by using nonlabelled cholesterol was performed in the diapausing prepupae of *Monema flavescens*. The state of prepupae was examined seven days after injection of each active fraction. The results are shown in Table I. By injecting the active fractions, diapausing prepupae began to change, as the *corpora allata* are active during the diapause period (Takeda, 1970). However, some of them became pupae according to the degree of the activity of the *corpora allata*. Further, it was shown that the activity of fraction 41 was always higher than that of fraction 51. Fractions 41 and 51 correspond to β-ecdysone and α-ecdysone, respectively. This is understood from the fact that β-ecdysone has more active hormonal actions than α-ecdysone. On the contrary, fractions 41 and 51 obtained from the prothoracic glands of diapausing

prepupae did not show any biological activities. In the control group using ethanol, biological activities were not found.

Discussion

The fine structure of prothoracic glands containing the developed agranular-ER, mitochondria and lipid droplets is different from that of the glands actively synthesizing the hormone. Well-developed agranular-ER, which may participate in the synthesis of the secretory substances, was found in the prothoracic glands of *Monema flavescens*. King, Aggarial and Bodenstein (1966) have also noticed a considerable increase in smooth-walled vesicles and agranular-ER in the ring gland of *Drosophila melanogaster*. In other insects, agranular-ER was also apparently often found (Locke, 1970). However, they are not always active with relation to ecdysone secretion (Herman, 1967). In rats, the adrenal cortex (Savatini, Robertis and Pleichmar, 1962) and interstitial cells (Christensen and Fawcett, 1961, 1966; Christensen, 1965) that synthesize steroid hormones contain a developed agranular-ER. It is suggested that there is a mutual relationship between the amount of agranular-ER and the amount of steroid, and, further, well-developed agranular-ER is the synthetic site of steroid hormone. In the lutein cells of the rat, agranular-ER is decreased by hypophysectomy and is increased by the supply of the tropic hormone (Enders and Lyons, 1964). Agranular-ER found in the prothoracic glands seemed to be the reaction site for side-chain alteration and hydroxylation of the cholesterol molecules in the course of the elaboration of ecdysone.

Agranular-ER, closely associated with mitochondria, is usually found in the prothoracic glands of *Monema flavescens* before synthesis of the secretory substances. The close relationship between the tubules of agranular-ER and the mitochondria seems to be related to the transference of cholesterol and other substances necessary for steroidgenesis. In some lepidopteran insects such as *Antheraea perni* and *Bombyx mori,* Beaulaton (1964, 1968) also observed the close relationship between the tubules of agranular-ER and macromitochondria. Copeland and Dalton (1959) also observed an association between agranular-ER and mitochondria in the cells of the pseudobranch gland of a teleost fish.

All the mitochondria in the prothoracic glands were spherical and their cristae were in the form of ducts. After contact with agranular-ER, some inclusions occasionally appeared in the mitochondria in the course of their transformation. Wilde (1969) presented schematic drawings of the mitochondrial transformation in the adult Colorado beetle, *Leptinotarsa decemlineata*. This resembled the mitochondria in the prothoracic glands of *Monema flavescens*. Such inclusions have been reported in mammalian steroid-secreting cells such as the testicular interstitial cells (Christensen and Fawcett, 1961, 1966), lutein cells (Enders, 1962; Enders and Lyons, 1964), and adrenocortex (Savatini *et al.,* 1962; Sheridian and Berst, 1967). Electron-dense globular granules and irregular droplets which appeared in the mitochondria might also be concerned with the synthesis of steroid hormone. The sequential transformation of the mitochondria in the prothoracic glands of some lepidopteran insects was investigated by Beaulaton (1968). He showed that essential modification which takes place during each intermolt effects the chondriosome of secretory cells, and he regarded the mitochondria as the initiating site of ecdysone synthesis. Waku and Sumimoto (1969) also regarded the mitochondria

in alluring glands of *Bombyx mori* as the starting organelles of the production of secretory substances. Agranular-ER and mitochondria are here considered to be the organelles related to the synthesis of ecdysone.

Lipoidal substances were also found in the prothoracic glands as found in other steroid-secreting cells. The lipid droplets found in the steroid-secreting cells were considered to be the lipid soluble hormones. However, lipid droplets were not synthesized in the testicular interstitial cells of opossum (Christensen, 1965). The lipid droplets in the prothoracic glands may be the stored form of cholesterol which is utilized for the synthesis of ecdysone.

One of the most striking characteristics of the prothoracic glands of *Monema flavescens* is the extreme irregularity of nuclear surface during secretory activity. The nuclear folding may be interpreted as the induction of the transport of the nuclear materials into the cytoplasm. In the prothoracic glands, the irregular outline of the nucleus may be one of the essential characteristics for the synthesis of these secretory substances. It is assumed that the irregular folding of the nuclear surface is necessary for the nucleo-cytoplasmic interaction, by increasing surface area. In the prothoracic glands of *Hyalophora cecropia,* an extremely irregular nucleus was found in the diapausing period (Herman, 1967). At the same time as the nuclear folding, tritium-labelled uridine moves out from the nucleus to the cytoplasm. The synthesis of RNA did not increase in the prothoracic glands of diapausing stage until the brain hormone activated the prothoracic glands to secrete ecdysone. When the glands were switched from the latent state to the active state, this led to an increase in the rate of RNA synthesis, suggesting that the prothoracic glands require RNA synthesis before the synthesis of ecdysone. This nascent RNA seems likely to be utilized to produce enzymes for the synthesis and the secretion of ecdysone. The correlation between the onset of RNA synthesis and the cytological changes was a striking feature. Similar results were also obtained in the prothoracic glands of *Antheraea polyphemus* (Oberlander, Berry, Krishnakumaran and Schneiderman, 1965).

Regarding the release of the secretory substances from the prothoracic glands, there are no earlier investigations. In *Hyalophora cecropia,* a thick peripheral membrane surrounds each cell and sends numerous invaginations into the gland cell cytoplasm (Herman, 1967). On the contrary, in *Monema flavescens,* the cell membrane showed a form of microvilli. In the releasing stage, the large secretory granules degenerated into many small particles. In *Papilio xuthus,* the secretory granules in the prothoracic glands are known to become a colloid for release through the cell membranes (Yashika and Yoshizaki, 1967). In *Monema flavescens,* these collapsed secretory granules containing ecdysone were released from expanded tips of the microvilli. This extrusion corresponds to Type III as proposed by Kurozumi and such phenomenon are usually found in the choroid plexus, the carpal organ of the pig and the interhepatic bile duct (Kurozumi, 1961).

The present results provide direct evidence of ecdysone biosynthesis in the prothoracic glands of *Monema flavescens.* Since the preliminary report that the prothoracic glands secrete ecdysone *in vitro* have appeared (Takeda, 1972a), many investigators have tried similar experiments independently. The metabolism of cholesterol in the ecdysal glands was also studied in the Y-organ of the crab, *Hemigrapsus nudus* (Spaziani and Kater, 1973). They investigated the uptake and turn-

over of ^{14}C-cholesterol in the Y-organ and obtained labelled "ecdysone-like metabolites," β-ecdysone, at 24 hours after injection of labelled cholesterol. Galbraith *et al.* (1970) showed that 7-dehydrocholesterol, which is found in relatively high concentration in insect prothoracic glands (Robbins, Thompson, Kaplanis and Shortino, 1964), is not incorporated into β-ecdysone in *Calliphora stygia* with significantly more efficiency than is cholesterol itself. In *Musca domestica,* Studinger and Willig (1975) reported that the radioactivity of labelled cholesterol is concentrated in some parts of the fat body and especially in the oenocytes. They postulated that the oenocytes play a central role in the metabolism and storage of sterols. King *et al.* (1974) cultured the prothoracic glands of *Manduca sexta,* examined the secretory substance and determined it to be α-ecdysone. Chino *et al.* (1974) also identified the secretory substance as α-ecdysone by using the prothoracic glands of *Bombyx mori, in vitro.* Similar results were also obtained in *Leucophaea maderae* (King and Marks, 1974; Borst and Engelmann, 1974), and *Tenebrio molitor* (Romer *et al.,* 1974).

In the ligature experiments of post-diapausing prepupae of *Monema flavescens,* the posterior part without the prothoracic glands also showed the ability of ecdysone biosynthesis as well as the anterior part with the prothoracic glands. When the prepupae were ligated, ecdysone had already been secreted. Therefore, ecdysone biosynthesis found in the posterior part seemed to be the result of some substances contained in the peripheral tissues or secreted from the prothoracic glands with ecdysone. In a preliminary report (Takeda, 1972a) the possibility was suggested that the active prothoracic glands secrete some enzymes or related substances which catalyze and elaborate one or more steps necessary for ecdysone biosynthesis. Nakanishi *et al.* (1972) have evidence which suggests that α-ecdysone can be synthesized from cholesterol and is converted into β-ecdysone in isolated abdomens of *Bombyx mori* without the prothoracic glands. Gersch and Stürzebecher (1971) demonstrated that ecdysone biosynthesis occurs in the isolated abdomens of *Mamestra brassicae.* Further, the abdomens of *Musca domestica* are also shown to synthesize α- and β-ecdysone (Studinger and Willig, 1975). Ecdysone biosynthesis outside the prothoracic glands can result from some enzymes or related substances secreted from the prothoracic glands with ecdysone in their former active stage.

The present results are explained as follows with special reference to ecdysone synthesis. Initial secretory activity of the prothoracic glands is characterized by increased cell and nuclear volume. The information necessary for the synthesis of secretory substances is transferred from the nucleus to the cytoplasm by the nuclear folding. Cholesterol molecules are then incorporated into the cytoplasm, transferred from agranular-ER to the mitochondria, and incorporated into the secretory substances of the mitochondria. In the course of this transference, cholesterol molecules are elaborated to the precursor of ecdysone. Some granules which are contained in the agranular-ER and the mitochondria and are able to synthesize ecdysone from cholesterol also consist of secretory substances. After breaking the mitochondrial sheath, the secretory substances collapse in the cytoplasm and are released through microvilli to the hemolymph. Just before the secretion, the precursor of ecdysone is elaborated to α-ecdysone and is secreted into the hemolymph where α-ecdysone is converted to β-form.

The author wishes to express his thanks to the late Prof. M. Fukaya for his continuous guidance and encouragement throughout the course of these experiments.

Summary

The problem of hormonal secretion with special reference to the synthesis of ecdysone in the prothoracic glands of *Monema flavescens* (Lepidoptera) was investigated.

Initial secretory activity of the prothoracic glands is characterized by increased cell and nuclear volume, and extensive nuclear folding. Cholesterol molecules are incorporated into the cytoplasm. They are transferred from the agranular-ER to the mitochondria and incorporated into the secretory granules of the mitochondria. In the course of transference, cholesterol molecules are elaborated to the precursor of ecdysone. After breaking the mitochondrial sheath, the secretory material breaks down in the cytoplasm and is released through microvilli to the hemolymph. At that time, the precursor of ecdysone is elaborated to α-ecdysone and is then secreted into the hemolymph where α-ecdysone is converted to the β-form.

In the present experiments, the prothoracic glands were demonstrated to synthesize ecdysones from cholesterol and secrete them. These facts were demonstrated both *in vivo*, and *in vitro*, in cultured prothoracic glands.

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